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RHEOTAXIS AND CHEMORECEPTION IN THE FRESHWATER SNAIL *BIOMPHALARIA GLABRATA* (SAY): ESTIMATION OF THE MOLECULAR WEIGHTS OF ACTIVE FACTORS

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For many aquatic organisms chemoreception plays an important, often decisive role in the selection and location of diet items (for reviews see Kohn, 1961; Lenhoff and Lindstedt, 1974; Bardach, 1975). In many instances it has proved possible, using a suitable behavioral or physiological assay, to identify some of the chemicals which elicit these responses (see for example Carr, 1967; Carr and Chaney, 1976; Gurin and Carr, 1971; Hara, 1976, 1977; Lenhoff, 1968, 1969; Suzuki and Tucker, 1971; Pawson, 1977). Indeed, from a study of the relationships between chemical structure and biological activity Hara (1976, 1977), working with the rainbow trout, *Salmo gairdneri*, and Lenhoff (1968, 1969), with the marine hydra, *Hydra littoralis*, have been able to describe in some detail the physico-chemical properties of the receptor sites themselves. The long term object of the present investigation has been to arrive at a similar level of understanding of the chemosensory mechanisms employed by *Biomphalaria glabrata* (Say) (Planorbidae, Mollusca), a phytophagous freshwater snail found in South America, and parts of the Caribbean.

The need for a detailed study of chemoreception in this organism is of particular importance, for two reasons. First, while freshwater plants are known to be an important factor conditioning the habitats of many species of freshwater snails (Gaevskaya, 1969; Bovbjerg, 1965; Pimentel and White, 1959; Pip and Stewart, 1976) little is known about the chemosensory basis of such interactions. *Biomphalaria glabrata* is particularly suitable for such a study for it has been shown to orient both chemotactically (Etges, 1963a, b; Michelson, 1960; Townsend, 1973a, b, 1974) and rheotactically (Etges and Frick, 1966) to dilute solutions of various plant extracts. Secondly, many species of freshwater snails are of considerable economic and medical importance as intermediate hosts of digenetic trematodes parasitic in man or domestic animals. *Biomphalaria glabrata*, for example, is host to the human schistosome, *Schistosoma mansoni* (Sambon). Attempts to control the disease usually rely on the use of molluscicides to remove or reduce snail populations in areas where the risk of transmission is particularly

high (Webbe and Jordan, 1966; Farooq, 1973). There are, however, numerous problems associated with the use of conventional molluscicides, among these being their high cost (Berg, 1973; Ritchie, 1973) and ecological side effects (Shiff and Garnett, 1961; Ritchie, 1973). A detailed knowledge of the role played by chemoreception in determining the distribution of snails within habitats may lead to more efficient and acceptable methods of control. For example, if baits could be formulated which could lure the snails to sites where slow release molluscicides were present, a saving in both labor and cost might be achieved (Etges, 1963a, b; Cardarelli, 1977).

In this paper a preliminary study of the molecular weight characteristics of chemicals eliciting rheotaxis is described.

Materials and Methods

Methods

Reproductively mature specimens of a Venezuelan albino strain of *Biomphalaria glabrata*, weighing 400 ± 30 mg ($\bar{x} \pm$ s.d), were selected from laboratory cultures maintained in the manner described by Thomas (1973). These individuals were then kept in water (see below) at densities of 20/liter in small plastic buckets, maintained at 26° C, under a constant (12L : 12D) regime. Each container was thoroughly cleaned out each day and fresh water (ionic composition $KHCO_3$—0.037 mM; KNO_3—0.0495 mM; $NaHCO_3$—0.634 mM; $MgSO_4$—0.13 mM; $CaCl_2$—2 mM; preaerated to pH 7.8–8.2) and food (0.1 g fresh lettuce per snail per day) provided. Each snail was used in no more than one trial per day, and was conditioned in clean water for one hour before the test.

Test apparatus and assay procedure

The test arena consisted of a shallow perspex trough ($24 \times 24 \times 3$ cm) into which a translucent polythene cylinder (23 cm diameter $\times$ 3 cm deep) was fitted. The substrate was formed by a glass plate which could be removed, and thoroughly cleaned before each trial. Snails placed at the center of this plate were thus free to move within a cylindrically symmetric environment.

To create the flow, two jets of compressed air were directed in opposite directions around the edges of the container from a single T-tube held close to and parallel with the surface. The net effect was to draw a stream of water approximately 12–15 cm wide and with surface speed 0.75–1.5 cm/sec across the center from the opposite side of the arena. Flow was returned via the sides.

Only snails which were actively moving at the bottom of their buckets at the time of their test were used in this study. This was done in order to standardize, as far as was possible, the initial behavioral state of the tested individuals. Each snail was removed gently from its container and then held just above the center of the glass plate floor until the foot extended and adhered to it. Subsequent movement of the snails was plotted on tracing paper on the screen of a video monitor. This was connected to a camera observing the movement of the snail from beneath the arena.

All tests were conducted under conditions of diffuse illumination from above. In order to counteract any residual directional bias due either to phototaxis (Sodeman, 1973; Sodeman and Dowda, 1974) or starting configuration (shell orientation/flow direction) snails were always started with the shell parallel to a fixed axis, while the direction of the current was rotated, periodically, through 90°

Stimuli

Lettuce was obtained from local market gardens. Care was taken to ensure that no plants had been treated with pesticides in the interval two weeks before use. Commercially available wheatgerm (Jordan's Natural Wheatgerm, Holme Mills, Biggleswade, Bedfordshire) was used. All plant extracts were made on the eve of an experiment using a standard technique.

In the case of lettuce, 20 g (wet weight) of the outer leaves were homogenized for one minute in a laboratory blender in 50 ml of distilled water chilled to 50° C. This was filtered through a Whatman No. 1 paper, made up to 100 ml with more distilled water and centrifuged at 10,000 rpm for 30 minutes. The supernatant was then filtered through Whatman glass fiber papers GF/C and GF/F (nominal cut-off 0.7 μm) and stored at 5° C until the following day.

Wheatgerm extract was made using the same technique, but here the working strength was 1 g (dry weight)/100 ml, and the first filtering stage was omitted.

Ultrafiltrates were prepared using Amicon Diaflo® ultrafiltration membranes UM–05, UM–2, UM–10, PM–30, and XM–100A. According to Amicon Corporation (1974) these membranes retain microsolutes of molecular weights (mol wt) greater than 500, 1000, 10,000, 30,000, and 100,000, respectively. Although retention is actually a function of molecular size, configuration and charge, a nominal retention characteristic curve can be drawn for each membrane, and these are shown in Figure 1.

Ultrafiltration was carried out at 5° C under nitrogen (20–40 psi) using a 100 ml stirred cell. All membranes were flushed *in situ* with 200 ml distilled water before use. One hundred ml of extract was prepared as described above and passed through the cell overnight.

Stimulus solutions were made up immediately before use as standard extract dilutions of 750 ml of water preheated to 26° C. This filled the arena to a depth of approximately 1.5 cm. As soon as the flow pattern had stabilized, the test was begun. Controls were run in water to which no extract had been added. In order to prevent contamination, stimuli were used for only one test and then discarded, the apparatus being thoroughly rinsed between tests to remove any residual traces of chemical.

Statistics

All trails representative of a particular treatment were superimposed on tracing paper so that their starting positions coincided, and the flow directions were parallel. The individual trails were then scored in the following manner.

The overall trial direction was defined as the angle made between the flow direction and a vector joining the starting point and the place where the trial first cut an 8 cm radius boundary centered on the middle of the arena. These

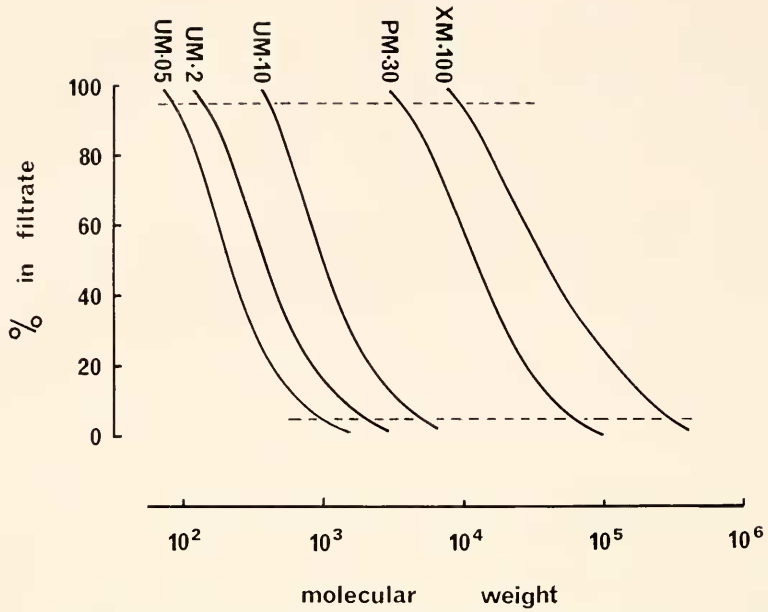

FIGURE 1. Molecular weight transmission charateristics of five Amico Diaflo® ultrafiltration membranes used in this paper. Data plotted in semi-logarithmic form. The ordinate shows the percentage of solute of given molecular weight transmitted by the membrane. Levels of the transmission (95% + 5%) are indicated by dotted lines. [*Replotted from Amicon Corporation publication* (1974, see Literature Cited).]

angles were measured to 50° accuracy using an anticlockwise convention and the flow direction as reference.

Test control data distributions were compared using the nonparametric tests for directional data developed and described by Watson (1962) and Mardia (1972). Overall estimates of the responses were obtained by treating each datum as a unit vector and calculating the resultant r (r, θ), where

$$\text{data set} = (\theta_i); \quad i = 1 \text{ to } n$$

$$r = \left[\left(\sum_{i=1}^{n} \sin \theta_i\right)^2 + \left(\sum_{i=1}^{n} \cos \theta_i\right)^2\right]^{\frac{1}{2}}/n$$

$$\theta = \tan^{-1}\left[\sum_{i=1}^{n} \sin \theta_i / \sum_{i=1}^{n} \cos \theta_i\right]$$

However, for the purpose of constructing graphs the scalar quantity r cos θ is more useful. This has a range of values from +1 to −1 and by convention has been taken as positive when the net movement is upstream (positive rheotaxis) and negative when the net movement is downstream (negative rheotaxis).

RESULTS

Typical data from a series of experiments in which lettuce extract (cultivar *Renate*) was tested are shown in Figure 2. The data are shown in two forms.

To the top, the superimposed trails obtained for a given treatment are shown. Each trail is from a different snail, and in each case the movement was centrifugal, with current direction from 6 o'clock to 12 o'clock. It can be seen that lettuce extract tested at a concentration of 10 ml/liter produces a strong polarization in favor of upstream movement (positive rheotaxis), whereas the control snails exhibited very little directional bias. The difference between these two distributions was highly significant [$P < 0.001$, Watson's U^2 test, (Watson, 1962)]. Below these trails the same data are re-represented in the form of a circular histogram.

Figure 2 also shows what effect passing the extract through ultrafiltration membranes has on the activity of the solution. As can be seen the effect of membranes PM–30, UM–10, UM–2 and UM–05 was to produce a gradual reduction in the length of the resultant vector and an increase in scatter in the individual trail directions as the retention characteristic moved to progressively lower molecular weights. At this concentration (10 ml extract/liter) all the treatments produced positive responses which were significantly different from the controls [PM–30, UM–10, $P < 0.01$; UM–2, UM–05, $P < 0.05$; Watson's U^2 test (Watson, 1962)]. The activity of the UM–05 and UM–10 filtrates, however, was significantly less ($P < 0.01$ and 0.05, respectively) than that of the original extract.

The reason why membranes with such widely different characteristics merely produce a gradual, rather than an all-or-nothing effect on activity can be seen immediately from Figure 1. It is clear that there is a considerable overlap between the characteristics of the four membranes concerned. For any individual membrane the 5–95% transmission limits span a molecular weight range equivalent to at least one order of magnitude. Consequently, in order to be able to interpret the results of ultrafiltration, some method of calculating the attenuation produced by any given membrane must be found.

In Figure 3 the response magnitude-extract concentration profile for *Renate* lettuce has been plotted in semi-logarithmic form. Other forms of representation are possible, but this method was found to be the most successful for the purposes of linearizing the data (see Beidler, 1971). From the linear regression on these points, it is possible to calculate the concentration of lettuce extract which would produce a response equivalent in magnitude to the response produced by a given filtrate. For example, UM–10 filtrate tested at concentrations of 2 ml/liter produces a response which is equivalent in magnitude to that of lettuce extract tested at a concentration of only 0.4 ml/liter. Thus the UM–10 filtrate only contains 20% of the original activity. From Figure 1 it can be seen that the point of 20% transmission occurs for molecular weights of approximately 2000. The results of testing four different ultrafiltrates of *Renate* extract, each at two different concentrations, are shown in Figure 3 (see legend), and the molecular weight estimates obtained shown in Table IA. The range of this extract was 1000–10,000. However, since it has been shown elsewhere (Carr, Hall and Gurin, 1974) that stimulants from different sources may be characterized by different molecular weight spectrums, these results have been complemented with tests using a different cultivated variety of lettuce and with wheatgerm. Data from experiments involving lettuce (cultivar *Amanda*) are

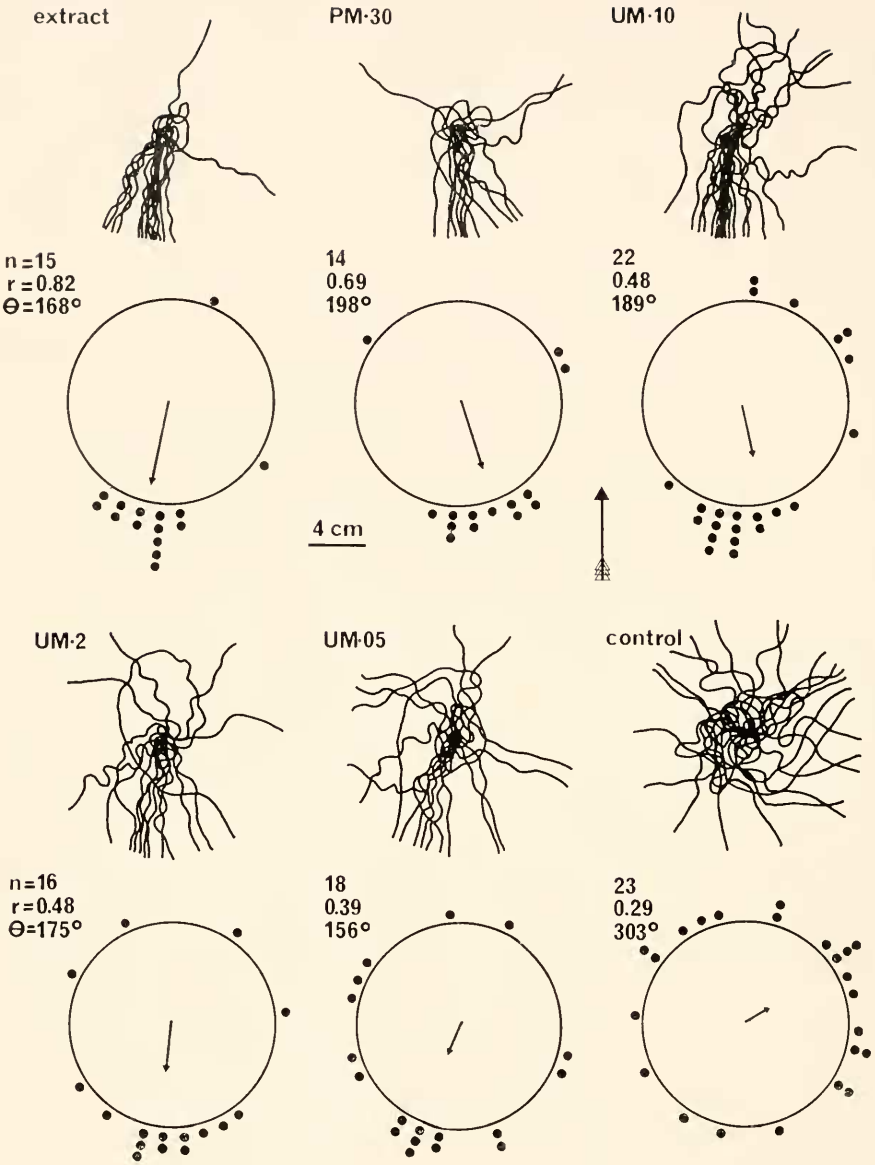

FIGURE 2. Trail data obtained using lettuce extract (cultivar *Renate*) and the effect produced by passing it through Diaflo® membranes. All stimuli were tested at concentrations of 10 ml extract or ultrafiltrate per liter. Control values were obtained using water alone. Each data set is shown in two forms. At the top the trails, starting at the center and radiating outward, of all the snails (n) tested in a given solution are shown superimposed. Below the same, data are represented in the form of a circular histogram. The vector in the center (r, θ) represents the magnitude of the resultant of the individual unit vectors obtained for each trial. A vector reaching the edge of the circle signifies a case in which all the snails

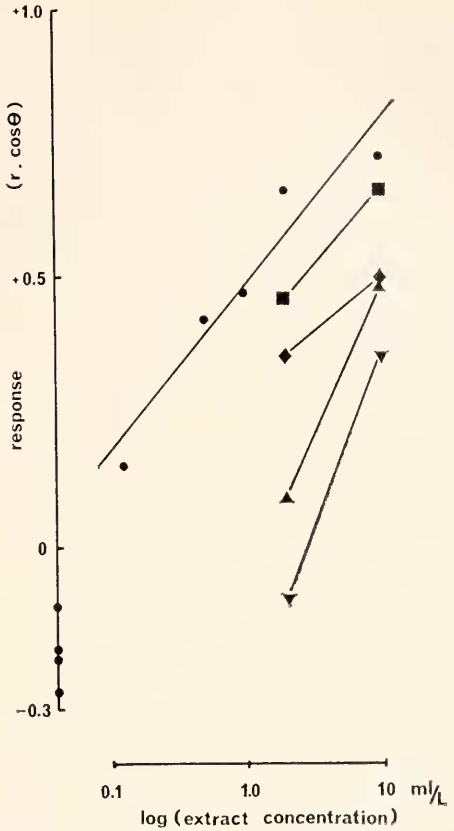

FIGURE 3. Response-concentration profile (circles) obtained for lettuce extract (cultivar *Renate*), together with the effects produced by various ultrafiltration treatments (other symbols). The projection of the resultant vector on the direction of flow (r cos θ) is used as an index of the response magnitude (see Methods). The regression equation for the lettuce extract data is r cos $\theta = 0.306$ log (concentration) $+0.48$. Each extract data point represents the resultant of 35–45 individual trials. Ultrafiltrate and control data points are averages of 15–25 trials. Squares represent PM–30 filtrate; diamonds, UM–10 filtrate; triangles, UM–2 filtrates; and inverted triangles, UM–50 filtrate. All filtrates were tested at concentrations of 2 ml and 10 ml/liter. Control values are shown on the ordinate.

also shown in Table IB. Exactly the same procedure was used here. The estimates arrived at using this extract agree well with those obtained using the *Renate* variety (Table IA). The range of values obtained was from 1000 to 6000. In contrast, however, estimates for the molecular weights of the attractants

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moved in the same direction. On the other hand, a vector of near zero length represents a case in which there was no bias and the direction of movement tended to be random. In each case the direction of flow is from 6 o'clock to 12 o'clock. Note the reduction in flow vector length and in the clustering of the trails which occurs as increasingly more retentive membranes are used.

TABLE I

The responses obtained for various ultrafiltrates of lettuce and wheatgerm extracts, together with estimates of the molecular weights of the attractants. The method of deriving the "estimated concentration" and "percentage of activity left" are explained in the text. Where the response to the filtrate was either very similar to that of the original extract, or was very small these values were not calculated, but a minimum or maximum molecular weight estimate is given. Asterisks denote values obtained using retentates. Note how these compare with those obtained using filtrates.

Stimulus	Concentration (ml/liter)	Response (r. cos θ)	Estimated conc. (ml/liter)	Per cent activity left	Estimated mol wt
(A) Lettuce (cultivar *Renate*)					
UM-05 filtrate	2	−0.10	—	—	>2000
UM-05 filtrate	10	0.35	0.40	4	1000
UM-2 filtrate	2	0.09	—	—	>3000
UM-2 filtrate	10	0.48	1.1	11	2000
UM-10 filtrate	2	0.35	0.40	20	2000
UM-10 filtrate	10	0.48	1.1	11	3000
PM-30 filtrate	2	0.46	0.80	40	10,000
PM-30 filtrate	10	0.66	4.0	40	10,000
(B) Lettuce (cultivar *Amanda*)					
UM-2 filtrate	3	−0.04	—	—	>3000
UM-2 filtrate	8	0.26	0.56	7	2000
UM-2 retentate	8	0.56	8.0	100	>3000*
UM-10 filtrate	3	0.11	0.16	5	6000
UM-10 filtrate	8	0.26	0.56	7	4000
UM-10 retentate	8	0.48	4.0	50	1000*
(C) Wheatgerm					
UM-2 filtrate	8	−0.12	—	—	>3000
UM-10 filtrate	8	0.29	0.1	1.25	>10,000
PM-30 filtrate	2	0.47	0.4	20	30,000
PM-30 filtrate	8	0.56	0.8	10	40,000
XM-100 filtrate	2	0.80	—	~100	~10,000
XM-100 filtrate	8	0.77	4	50	40,000

in wheatgerm were, on the whole, higher (mol wt 10,000–40,000) than those obtained using lettuce extract (Table IC).

DISCUSSION

Before proceeding with a discussion of the results, it is important to view critically the methods used in this study. Ultrafiltration has been used extensively in studies of chemoreception as a means of removing or selectively attenuating specific molecular weight fractions from solutions containing stimulants (Carr, Hall and Gurin, 1974; Carr and Gurin, 1975; Carr, 1976; Carr and Chaney,

1976). However, the "cut-off points" for each ultrafiltration membrane, in fact, span a considerable range of molecular weights (Fig. 1). Thus, even if the stimulant chemicals were all of the same molecular weight, a membrane whose "cut-off point" lay above that weight would not necessarily remove the biological activity completely. The situation is further complicated by the possibility that the "stimulant" may, in fact, be a group of chemicals all with different molecular weights.

While bearing in mind that the published characteristics of each membrane are at best nominal and depend on a number of factors such as molecular charge, configuration and the presence of other solutes, the first difficulty may be overcome by relating the activity of an ultrafiltrate to the stimulus concentration-response magnitude profile of the original extract. This provides an estimate of the alteration produced by the given membrane and consequently a value for the molecular weight of the attractant. The second difficulty, the possible presence of a range of active molecules with different retention characteristics, can be overcome, to some extent, by using a range of membranes with widely different ultrafiltration properties. Membranes whose 50% retention points lies above the mean molecular weight of the attractants will provide estimates of this mean which are too high. Conversely, membranes whose 50% retention points lie below this mean will produce estimates which are too low. In general this tendency for ultrafiltration membranes with high molecular weight cut-offs to give higher estimates than those with low cut-offs is borne out by the results shown in Table I, although the effect is particularly obvious only for the wheatgerm data.

The results of this preliminary study of the characteristics of stimulants triggering rheotaxis clearly demonstrate that the factors involved are not simple compounds, such as amino acids, short chain organic acids or small sugars, but are substances having molecular weights in excess of 1000. An exact value is, however, for reasons given above, difficult to determine. While the estimates obtained for two varieties of cultivated lettuce are in agreement and provide a value somewhat below 10,000, all the estimates made for wheatgerm lie on or above this limit (Table I). It is unlikely then that the response is specific to a single chemical compound as has been found to be the case in some marine coelenterates (Lenhoff and Lindstedt, 1974). On the contrary, these differences suggest that some generalized property of a class of macromolecules is the active stimulus. It is interesting to note in this context that differences in stimulant molecular weights have also been found for the shrimp *Palaemonetes pugio* when tested with extracts made from a variety of marine invertebrates (Carr and Gurin, 1975).

In the past studies of chemoreception and food-finding behavior in aquatic organisms have stressed the role played by low molecular weight nitrogenous compounds. For example, sensitivities to amines and amino acids have been demonstrated in a number of marine and freshwater fish (for example Carr, 1976; Carr and Chaney, 1976; Hara, 1976, 1977; Pawson, 1977; Suzuki and Tucker, 1971), in marine Crustacea (Fuzessery and Childress, 1975; Laverack, 1963; Mackie, 1973), marine molluscs (Carr, 1967; Crisp, 1967; Jahan-Parwar, 1975) and a freshwater planarian (Coward and Johannes, 1969). Although in many instances the activity of food extracts is well accounted for by the presence

of these substances (see for example Carr, 1967, 1976; Carr and Chaney, 1976; Mackie, 1973; Pawson, 1977), it is becoming increasingly clear that compounds of larger molecular weights play an important stimulatory role in certain cases (see, for example, Ash, McClure and Hirsch, 1973; Carr, Hall and Gurin, 1974; Carr and Gurin, 1975; Gurin and Carr, 1971). For instance, Carr and his co-workers have shown that for the marine prosobranch, *Nassarius obsoletus*, macromolecules with properties consistent with those of proteins and peptides are the main active factors in extracts eliciting exploratory feeding behavior. In the fresh water planarian, *Dugesia dorotocephala*, the factors which elicit feeding behavior have molecular weights of between 700 and 2000 (Ash *et al.*, 1973).

Aquatic macrophytes and algae (Fogg, 1971; Hellebust, 1974; Wetzel and Manny, 1972) release large quantities of organic carbon into the surrounding water. It has been suggested that these chemicals may attract aquatic snails and be, in part, responsible for certain plant-snail associations observed in the field (Pip and Stewart, 1976). Natural plant exudates may also be responsible for the positive rheotactic movements which have sometimes been observed in field mark-recapture experiments performed with *B. glabrata* (Paulini, 1963; Pimentel and Ildefonson, 1957; Radke and Ritchie, 1961). They are certainly not simply responses to the presence of the currents themselves (Etges and Frick, 1966).

The majority of the material secreted by plants is made up of low molecular weight compounds such as glucose and glycollic acid (Hellebust, 1974; Wetzel and Manny, 1972) but polysaccharides, polypeptides and glycoproteins are also released (Fogg, 1971; Hellebust, 1974). These simpler compounds are, however, often photolabile and may be rapidly utilized by epiphytic organisms (Allen, 1976; Sepers, 1977; Wetzel and Manny, 1972). Since the rheotaxic response described here allows *Biomphalaria glabrata* to orient to distant sources of organic chemical, it is possible that such ecological pressures have favored the evolution of chemoreceptivity for larger, more stable molecules.

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Summary

1. Dilute solutions of lettuce and wheatgerm extracts trigger positive rheotaxis in the freshwater snail, *Biomphalaria glabrata*. This response can be used as the basis of a sensitive bioassay for characterizing and identifying the chemicals to which the snail is attracted.

2. Using ultrafiltration techniques a range of different molecular weight fractions could be attenuated or removed from these extracts. By comparing the activity of these solutions with that of the original extract an estimate of the molecular weight of the attractant could be made.

3. In both cases the molecular weights of the attractants were estimated as being greater than 1000. Those in the lettuce were estimated at lying between 1000

and 10,000; whereas for wheatgerm the values were slightly higher and lay between 10,000 and 40,000. The ecological significance of these results is discussed.

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